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Authors

Bistrup, A
Bhakta, S
Lee, JK
et al.

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Sulfotransferases of Two Specificities Function in the Reconstitution of High Endothelial Cell Ligands for L-selectin

Annette Bistrup,* Sunil Bhakta,[¶] Jin Kyu Lee,* Yevgeniy Y. Belov,* Michael Dee Gunn,[‡] Feng-Rong Zuo,[¶] Chiao-Chain Huang,[¶] Reiji Kannagi,[§] Steven D. Rosen,* and Stefan Hemmerich[¶]

*Department of Anatomy and Program in Immunology, and [‡]Cardiovascular Research Institute, University of California, San Francisco, California 94143; [§]Program in Experimental Pathology, Aichi Cancer Center, Nagoya, Japan; [¶]Clontech Laboratories, Inc., Palo Alto, California 94303; and [¶]Department of Respiratory Diseases, Roche Bioscience, Palo Alto, California 94304-1397

Abstract. L-selectin, a lectin-like receptor, mediates rolling of lymphocytes on high endothelial venules (HEVs) in secondary lymphoid organs by interacting with HEV ligands. These ligands consist of a complex of sialomucins, candidates for which are glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1), CD34, and podocalyxin. The ligands must be sialylated, fucosylated, and sulfated for optimal recognition by L-selectin. Our previous structural characterization of GlyCAM-1 has demonstrated two sulfation modifications, Gal-6-sulfate and GlcNAc-6-sulfate in the context of sialyl Lewis x. We now report the cloning of a Gal-6-sulfotransferase and a GlcNAc-6-sulfotrans-

ferase, which can modify GlyCAM-1 and CD34. The Gal-6-sulfotransferase shows a wide tissue distribution. In contrast, the GlcNAc-6-sulfotransferase is highly restricted to HEVs, as revealed by Northern analysis and in situ hybridization. Expression of either enzyme in Chinese hamster ovary cells, along with CD34 and fucosyltransferase VII, results in ligand activity, as detected by binding of an L-selectin/IgM chimera. When coexpressed, the two sulfotransferases synergize to produce strongly enhanced chimera binding.

Key words: sulfotransferase • carbohydrate • L-selectin • high endothelial venule • endothelium

SULFATION of carbohydrates attached to glycoproteins, glycolipids, and proteoglycans confers highly specific functions onto these macromolecules (Nelson et al., 1995; Hooper et al., 1996; Rosenberg et al., 1997). Examples of sulfation-dependent recognition phenomena are seen in diverse biological systems, including blood clotting, cytokine sequestration and receptor binding, control of the circulatory half-life of pituitary hormones, and plant-bacterial symbiosis. Therefore, the sulfotransferases that catalyze the sulfation of carbohydrates have become the focus of increasing research interest.

In recent years, the role of sulfated carbohydrates in leukocyte-endothelial interactions has been established (Kansas, 1996; Rosen and Bertozzi, 1996). During the process of normal lymphocyte recirculation, L-selectin on lymphocytes interacts with a set of sulfated glycoprotein ligands on the endothelial cells of high endothelial venules

(HEVs).¹ This interaction leads to lymphocyte rolling, which constitutes the first step in the recruitment cascade that culminates in the migration of the lymphocyte into the underlying tissue (Springer, 1994; Butcher and Picker, 1996). The involvement of L-selectin in leukocyte recruitment to certain sites of acute and chronic inflammation has also been demonstrated (Lewinsohn et al., 1987; Ley et al., 1991; Michie et al., 1993). The high endothelial cells (HECs) of HEVs within lymphoid organs and of HEV-like vessels that develop at sites of chronic inflammation have long been known to incorporate large amounts of [³⁵S]sulfate in several species (Andrews et al., 1982; Girard and Springer, 1995a). The functional significance of this

Address correspondence to Steven D. Rosen, University of California, Lung Biology Center, Box 0854, San Francisco, CA 94143-0854. Tel.: (415) 476-1579. Fax: (415) 206-4123. E-mail: sdr@itsa.ucsf.edu or Stefan Hemmerich, Department of Respiratory Diseases, Roche Bioscience, Palo Alto, CA 94304-1397. Tel.: (650) 354-7169. Fax: (650) 354-7554. E-mail: stefan.hemmerich@roche.com

1. *Abbreviations used in this paper:* BAC, bacterial artificial chromosome; C2GnT, core 2 β 1→6 N-acetylglucosaminyltransferase; C6/KSST, chicken chondroitin/keratan sulfate sulfotransferase; C6ST, human chondroitin-6-sulfotransferase; CM, conditioned medium; EST, expressed sequence tag; FTVII, fucosyltransferase VII; Gal, galactose; GlcNAc, N-acetylglucosamine; GlyCAM-1, glycosylation-dependent cell adhesion molecule 1; HEC, high endothelial cell; HEC-GlcNAc6ST, HEC GlcNAc-6-sulfotransferase (AF131235, AF131236); HEV, high endothelial venule; HPAEC, high pH anion exchange chromatography; HUVEC, human umbilical vein endothelial cell; KSGal6ST, keratan sulfate Gal-6-sulfotransferase; MFI, mean fluorescence intensity; nt, nucleotide(s); PE, phycoerythrin; RT, reverse transcriptase; sLe^x, sialyl Lewis x.

extensive sulfation was not understood until the ligands for L-selectin were elucidated.

To date, four discrete HEV-associated glycoprotein ligands for L-selectin have been identified at the molecular level: glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1) (Lasky et al., 1992), CD34 (Baumhueter et al., 1993; Puri et al., 1995; Shailubhai et al., 1997), mucosal addressin cell adhesion molecule 1 (MAdCAM-1) (Berg et al., 1993), and podocalyxin (Sasseti et al., 1998). At least one other component, Sgp200, remains to be cloned (Berg et al., 1991; Hemmerich et al., 1994b; Hoke et al., 1995). These glycoproteins all contain mucin-like domains and are heavily *O*-glycosylated (Puri et al., 1995). It is well established that optimal recognition of these ligands by the C-type lectin domain of L-selectin requires sialylation (Imai et al., 1991), fucosylation (Maly et al., 1996), and sulfation (Imai et al., 1993). The protein scaffolds underlying these ligands are also expressed in tissues other than HEVs, such as mammary gland, non-HEV endothelium, hematopoietic precursors, and kidney podocytes (Dowbenko et al., 1993; Baumhueter et al., 1994; Kershaw et al., 1997). However, the L-selectin-reactive glycoforms of these proteins are highly restricted to HECs (Dowbenko et al., 1993; Baumhueter et al., 1994; Sasseti et al., 1998).

To understand the posttranslational modifications that underlie recognition of ligands by L-selectin, we previously undertook the analysis of the oligosaccharides of GlyCAM-1 which was affinity purified with recombinant L-selectin (Hemmerich and Rosen, 1994; Hemmerich et al., 1994a, 1995). We detected two specific sulfated monosaccharides: *N*-acetylglucosamine-6-sulfate (GlcNAc-6-sulfate) and galactose-6-sulfate (Gal-6-sulfate), which were present in equal amounts. These sulfate modifications are found, respectively, within two capping structures based on sialyl Lewis x (sLe^x): sialyl 6-sulfo Le^x and sialyl 6'-sulfo Le^x (see Fig. 1 and Table I). The simplest chains of GlyCAM-1 consist of sialyl 6'-sulfo Le^x and sialyl 6-sulfo Le^x as extensions from an internal trisaccharide known as core 2 (see Fig. 1 and Table I). More complex structures with increased chain size and multiple sulfate esters comprise the majority of the chains and remain to be analyzed in detail.

The sulfate dependence for L-selectin binding of HEV ligands was first established in pharmacological experiments with metabolic inhibitors of sulfation, i.e., chlorate and brefeldin A (Imai et al., 1993; Hemmerich et al., 1994b; Crommie et al., 1995; Shailubhai et al., 1997). Additional evidence for the importance of sulfation has emerged from studies with two mAbs, MECA-79 and G72. MECA-79 stains lymph node HEVs and blocks L-selectin-dependent lymphocyte attachment to HEVs in vitro and in vivo (Streeter et al., 1988). This antibody also stains HEV-like vessels that develop at sites of chronic inflammation (Michie et al., 1993; Lee and Sarvetnick, 1994; Girard and Springer, 1995a; Onrust et al., 1996). MECA-79 reacts with all the L-selectin ligands described above (Imai et al., 1991; Berg et al., 1993; Hemmerich et al., 1994b; Sasseti et al., 1998). This broad reactivity is thought to reflect its binding to a posttranslational modification that is common to many L-selectin ligands (Berg et al., 1993; Hemmerich et al., 1994b). The presence of the MECA-79 epitope is currently accepted as a predictor of L-selectin ligand activ-

ity (Girard and Springer, 1995a). However, endothelial and leukocyte ligands for L-selectin exist that lack this epitope (Fuhlbrigge et al., 1996; Clark et al., 1998). Although the precise structure of the MECA-79 epitope has not been elucidated, it is critically dependent on sulfation but does not require sialic acid or fucose (Hemmerich et al., 1994b; Maly et al., 1996; Shailubhai et al., 1997). The mAb G72 appears to have a more complex epitope in that it reacts with synthetic sialyl 6-sulfo Le^x in a sialic acid- and sulfate-dependent manner (Mitsuoka et al., 1998). G72 stains HEVs in human peripheral lymph node intensely and blocks the binding of a human L-selectin/IgG chimera to HEV in vitro. Taken together, these results strongly implicate sulfated structures, including sialyl 6-sulfo Le^x, as important recognition motifs for L-selectin binding to HEVs.

The contribution of individual sulfate esters to L-selectin binding affinity has been examined in several studies using sulfated sLe^x structures or analogues thereof. Direct binding measurements and competition studies have yielded evidence for enhanced binding (compared with sLe^x) from the Gal-6-sulfate modification (Tsuboi et al., 1996; Koenig et al., 1997), the GlcNAc-6-sulfate modification (Scudder et al., 1994; Saunders et al., 1996; Galustian et al., 1997), and from both (Yoshino et al., 1997). Significantly, Bertozzi et al. (1995) found that several sulfated variants of lactose were equal to or more potent than sLe^x in inhibiting the binding of L-selectin to GlyCAM-1 in an ELISA. For example, 6',6-disulfo lactose (see Table I) competed twofold better than sLe^x, demonstrating that the relevant sulfate modifications by themselves, in the absence of any contribution from sialic acid or fucose, can confer a significant degree of binding to L-selectin.

In our search for the relevant sulfotransferases responsible for the synthesis of the specific sulfated carbohydrates on L-selectin ligands, Bowman et al. (1998) recently identified a GlcNAc-6-sulfate sulfotransferase activity in porcine organs. This activity is highly expressed in lymph node and spleen, relative to other tissues. Strikingly, it is greatly enriched in HECs, with only scant activity detectable in lymph node lymphocytes or HEC-depleted stromal cells. To identify candidate sulfotransferases at the molecular level, we probed expressed sequence tag (EST) databases for human sequences with homology to the cDNA sequence for a chicken Gal-6-sulfotransferase (Fukuta et al., 1995; Habuchi et al., 1996, 1997). Here, we describe the cloning of two cDNAs encoding carbohydrate sulfotransferases that can modify GlyCAM-1 and CD34. One enzyme is a Gal-6-sulfotransferase, which has been independently cloned by another group. The other is a novel enzyme exhibiting GlcNAc-6-sulfotransferase activity. The Gal-6-sulfotransferase has a wide tissue distribution, whereas the GlcNAc-6-sulfotransferase is highly restricted to HECs. Finally, we show that both enzymes can contribute to the generation of functional ligands for L-selectin and do so in a synergistic manner.

Materials and Methods

Semiquantitative Reverse Transcription PCR Analysis

HECs were purified from human tonsils as described previously (Sasseti

et al., 1998). Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics. Total RNA was isolated from HECs, HUVECs, and human tonsillar lymphocytes by lysis and extraction with RNAzol (Tel-Test, Inc.). Approximately 45 µg RNA was obtained from 7×10^6 cells. First strand cDNA was made from 2 µg total RNA primed with random hexamers using AMV reverse transcriptase (RT; Life Technologies, Inc.). PCR reactions were carried out in a total volume of 10 µl of 1× KlenTaq buffer (Clontech) containing 400 nM primers, 200 µM dNTPs, 0.2 µl KlenTaq Advantage DNA polymerase mix (KlenTaq polymerase; Clontech), and 1.0 µl of twofold serially diluted cDNA as template. Cycling conditions were as follows: 1 min at 94°C; 30 cycles of 30 s at 92°C followed by 1 min 15 s at 68°C; one cycle of 10 min at 68°C. The following primers were used: for HEC-GlcNAc6ST, 5'-AAACTCAAGAAGGAG-GACCAACCCTACTATGTGATGC-3' and 5'-GTGGATTGCTCA-GGGACAGTCCAGCTAGACAGAAGAT-3', which amplify a 456-bp fragment corresponding to nucleotides (nt) 884–1339 in Fig 3 a; for hypoxanthine phosphoribosyltransferase (HPRT), 5'-CCTGCTGGATTACATCAAAGCACTG-3' and 5'-TCCAACACTTCGTGGGGTCTCT-3'. The resulting amplified DNA was electrophoresed and visualized by ethidium bromide.

Preparation of an HEC cDNA Expression Library

A cDNA expression library was prepared from human HECs using the SMART cDNA technology (Clontech), which incorporates a long-distance PCR amplification step of first strand cDNA. 1 µg of total HEC RNA prepared as above was mixed with a modified oligo (dT) primer (1 µM) containing a NotI site and a universal site for 3' priming of the PCR reaction and the SMART oligonucleotide (1 µM), which provides a universal site for 5' priming of the PCR reaction. This mixture was heated at 72°C for 2 min to disrupt RNA secondary structure, and first strand cDNA was synthesized by Moloney murine leukemia virus (MMLV) RT in a total volume of 10 µl. 2 µl of this reaction mixture was subjected to 18 cycles of long-distance PCR primed by the universal primers using KlenTaq polymerase (Clontech). The PCR reaction mixture was incubated in the presence of proteinase K for 1 h at 45°C to destroy the KlenTaq polymerase activity, followed by heat inactivation at 90°C for 10 min. The double-stranded cDNA was polished by treatment with T4 DNA polymerase at 16°C for 30 min, followed by ligation to EcoRI/BstXI adaptors (Invitrogen) overnight at 16°C in the presence of T4 DNA ligase. Adaptor-ligated cDNAs were digested with NotI and then phosphorylated. The double-stranded cDNA was purified by size fractionation, ligated into EcoRI/NotI-digested pCDNA1.1 (Invitrogen), and introduced into *Escherichia coli* MC1061/p3 (Invitrogen) by electroporation. The library contained 500,000 independent clones with an average insert size of 1.1 kb.

Molecular Cloning of HEC-GlcNAc6ST

HEC-GlcNAc6ST (human) was cloned from the HEC cDNA library by modification of a pool selection procedure (Kolodkin et al., 1997). In brief, an aliquot (comprising 400,000 colony forming units) of the amplified bacterial stock of the HEC cDNA library was plated onto 200 LB plates and grown for ~18 h at 37°C. Each pool of 2,000 colonies was harvested and grown for an additional 2 h at 37°C, and glycerol stocks were made. PCR analysis was performed, using the HEC-GlcNAc6ST-specific primers described above, to identify positive pools. 1 of the 9 positive pools was titrated and plated onto 40 plates to yield 100 colonies per plate. These pools were expanded and analyzed as in the first round. A single positive subpool was titrated and plated onto 20 plates of 10 colonies each. Analysis of individual colonies by PCR resulted in a single positive clone, which was sequenced (Sanger et al., 1977). To clone the murine HEC-GlcNAc6ST, a 241-bp probe (nt 26–267) was amplified from the EST clone (AA522184; Research Genetics Inc., Huntsville, AL) and used as probe for screening a bacterial artificial chromosome (BAC) library from the C57BL/6 mouse (Genome Systems, Inc.). From the single positive clone, DNA was purified and sequenced directly, using primers derived from EST AA522184 (forward: 5'-TGGGTCAGCATGCCCTCCATAC-TAAC-3'; reverse: 5'-TTCTAAGATTCCGGTTGCTTCTCCGTGGAC-3') and then obtaining sequence upstream (1559 nt) and downstream (582 nt). The resulting 1926 nt sequence was confirmed by resequencing in both directions.

Molecular Cloning of KSGal6ST

A human fetal brain library (λ ZAP; Stratagene) was the kind gift of Dr. Marc Tessier-Lavigne at the University of California, San Francisco. Ap-

proximately 10^6 plaques were screened with a probe consisting of a 730-bp HindIII/BamHI restriction fragment from IMAGE Consortium clone 40604 (EMBL/GenBank/DBJ accession no. R55609) using standard techniques (Sambrook et al., 1989). 18 independent positive plaques were identified after the second round of hybridization. Cloned fragments were excised and sequenced as above.

Northern Blot Analysis

The probe for HEC-GlcNAc6ST consisted of a 496-bp fragment from LifeSeq clone no. 2620445, corresponding to nt 1021–1516 of the cloned cDNA (see Fig. 3 A). The probe was labeled with [α - 32 P]dATP (Amersham Pharmacia Biotech) by the random decamer priming method (Strip-EZ DNA kit; Ambion, Inc.). Multiple Tissue Northern blots (Clontech) containing poly(A)⁺ RNA from various human tissues were hybridized at 60°C overnight in Rapid-Hyb (Amersham Pharmacia Biotech) and then washed twice at room temperature for 15 min in 2× SSC/0.1% SDS followed by two 15-min washes at 60°C in 0.1× SSC/0.1% SDS and autoradiography. For the Northern blot to establish expression in HECs, poly(A)⁺ RNA was prepared from 1.5×10^7 HECs and 2.0×10^7 HUVECs, respectively. Isolation of the poly(A)⁺ RNA with oligo(dT) latex beads was performed according to the manufacturer's protocol (Oligotex Direct kit; QIAGEN, Inc.). Approximately 2 µg poly(A)⁺ RNA was loaded per lane. The RNA was separated by electrophoresis in a 1% denaturing agarose-formaldehyde gel and transferred to positively charged nylon filters (Hybond N+). The filters were hybridized and washed as for the Multiple Tissue blots. Blots were stripped using the Strip-EZ DNA kit (Ambion, Inc.), according to the manufacturer's protocol.

In Situ Hybridization

Paraffin sections (5 µM) from C57BL/6 mice were deparaffinized, fixed in 4% paraformaldehyde, and treated with proteinase K. After washing in 0.5× SSC, the sections were covered with hybridization solution (50% formamide, 300 mM NaCl, 20 mM Tris, pH 8.0, 5 mM EDTA, 1× Denhardt's, 10% dextran sulfate, 10 mM DTT), prehybridized for 1–3 h at 55°C, and hybridized overnight with sense or antisense 35 S-labeled riboprobe transcribed from the IMAGE consortium clone 851801 (EMBL/GenBank/DBJ accession no. AA522184; Research Genetics, Inc.) which had been modified by digestion with SacI followed by religation. After hybridization, sections were washed at high stringency, dehydrated, dipped in photographic emulsion NTB2 (Eastman Kodak Co.), stored at 4°C for 2–8 wk, developed, and counterstained with hematoxylin and eosin.

Sulfation of GlyCAM-1/IgG in COS Cells

For generation of recombinant GlyCAM-1/IgG fusion protein, COS-7 cells were grown to 80% confluency in a T162 culture flask (Corning-Costar) and transfected with 8 µg of a plasmid encoding GlyCAM-1/IgG and 8 µg of plasmid encoding either HEC-GlcNAc6ST (pCDNA1.1), KSGal6ST (pCDNA3.1), or the empty vector (pCDNA1.1) using Lipofectamine (Life Technologies) in Opti-MEM (Life Technologies). The cDNA encoding the GlyCAM-1/IgG chimera was constructed by amplifying the entire coding sequence of murine GlyCAM-1 (Lasky et al., 1992) by PCR and cloning the resulting fragment into the pIG1 vector (Simmons, 1993). Cells were grown for 12 h after transfection in DME containing 10% FBS, then cell layers were washed once with PBS and cultured for 72 h in serum-free medium (Endothelial SFM, 16 ml per flask; GIBCO BRL) supplemented with Na₂³⁵SO₄ (0.25 mCi/ml, 1,400 Ci/mmol; ICN). Recombinant GlyCAM-1/IgG fusion protein was isolated from the conditioned medium (CM) by affinity chromatography on protein A-agarose (Watson et al., 1990). The protein was then transferred into 50 mM ammonium bicarbonate on a Centricon 30 concentrator (Amicon, Inc.). 1% of each sample was analyzed by SDS-PAGE, and the remaining samples were lyophilized for subsequent acid hydrolysis and positional analysis.

Analysis of Sulfated GlyCAM-1/IgG Carbohydrates

The lyophilized recombinant GlyCAM-1/IgG samples were hydrolyzed in 0.2 M H₂SO₄, and the hydrolysates were prepared for analysis by high pH anion exchange chromatography (HPAEC) essentially as described previously (Hemmerich et al., 1994a), with the following modifications: (a) after 30 min hydrolysis in 0.2 M H₂SO₄ at 96°C, and before the initial gel filtration step, excess sulfate was precipitated by addition of an equivalent amount of Ba(AcO)₂; (b) the second ion exchange column (DEAE-

Sephacrose) was equilibrated in 2 mM pyridine-acetate, pH 5.0, and eluted with a gradient of 2–500 mM pyridine-acetate. The monosulfated oligosaccharides eluted from this column between 50 and 250 mM pyridine acetate. The eluate was lyophilized, redissolved in 100 μ l of water, and 30- μ l samples of the resulting solution were subjected directly to HPAEC. HPAEC was performed using a Dionex DX 500 chromatographic analysis system and a Carbowac 1 column (Dionex Corp.) essentially as described (Hemmerich et al., 1994a, 1995), with column elution performed isocratically with 150 mM NaOH in 400 mM NaOAc at 1 ml/min. The authentic carbohydrate standards used in this analysis were obtained as described (Hemmerich et al., 1994a, 1995).

Flow Cytometry

CHO/FTVII/C2GnT cells were grown to 80% confluency in T75 flasks (6×10^6 cells per flask; Nalge Nunc) and then transfected with plasmids encoding C2GnT (1 μ g, pCDNA1.1), fucosyltransferase VII (FTVII; 1 μ g, pCDNA3.1), human CD34 (2 μ g, pRK5), sulfotransferases (at the concentrations indicated for each experiment), and vector plasmid (pCDNA3.1) to achieve 8 μ g total DNA per flask, using Lipofectamine. 48 h after transfection, the cells were harvested in 0.6 mM EDTA in PBS without calcium and magnesium, washed once in assay buffer (0.1% BSA in PBS), and resuspended at 4×10^6 /ml in assay buffer. 25 μ l of this cell suspension was added to wells of 96-well plates containing 50 μ l of L-selectin/IgM chimera, or assay buffer. Cells were incubated on ice for 30 min, washed twice in assay buffer, and resuspended in 50 μ l assay buffer containing secondary staining reagents. Cells were incubated for another 30 min, washed twice in assay buffer, and then resuspended in 50 μ l assay buffer containing tertiary staining reagents and/or directly conjugated primary mAbs. For the sialyl 6-sulfo Le^x or overall sLe^x determinations, cells were incubated with G72 mAb or HECA 452 (PharMingen), respectively, diluted in assay buffer, and then reacted with rabbit anti-mouse IgG-FITC (Zymed Laboratories, Inc.) (for G72) or mouse anti-rat IgM-FITC (PharMingen) (for HECA 452). The cells were incubated for 30 min, washed twice, and fixed in 1.5% paraformaldehyde in PBS, pH 7, for 20 min before transfer into 300 μ l of assay buffer. Cells were analyzed on a FACSortTM (Becton Dickinson) flow cytometer using CELLQuest software (Becton Dickinson). To produce the L-selectin/IgM chimera (Maly et al., 1996), COS cells (10-cm plates) were transfected with L-selectin/IgM cDNA in pCDM8 (8 μ g cDNA, 50 μ l Lipofectamine per plate) and incubated in Opti-MEM for 10 d, at which time the CM was harvested and clarified by centrifugation. The CM was concentrated to half its original volume, titered, and used neat. Biotinylated goat anti-human IgM (μ) and streptavidin-FITC were purchased from Caltag Laboratories. The L-selectin mAb Mel-14 (Gallatin et al., 1983) was purified by ammonium sulfate precipitation from hybridoma cell culture supernatant, followed by immunoaffinity purification with mouse L-selectin/IgG. The control for Mel-14 was a rat IgG2a myeloma (Zymed Laboratories, Inc.). For the two-color analysis, we used mouse anti-CD34 phycoerythrin (PE) (QBend10-PE; Coulter Corp.) and the isotype-matched control, mouse IgG1-PE (Caltag Laboratories).

Results

Identification of Two cDNAs Predicting Carbohydrate Sulfotransferases

Our previous structural analysis of the carbohydrate chains of GlyCAM-1 indicated that the Gal-6-sulfate and GlcNAc-6-sulfate modifications accounted for essentially all of the sulfation of GlyCAM-1 (Hemmerich et al., 1994a). The previously cloned chicken chondroitin/keratan sulfate sulfotransferase (C6/KSST) has been shown by Habuchi et al. (1997) to catalyze sulfation at C-6 of galactose in Sia α 2 \rightarrow 3Gal β 1 \rightarrow 4GlcNAc, which is a core structure within the capping groups shown in Fig. 1. We used the cDNA sequence encoding C6/KSST to probe the National Center for Biotechnology Information (NCBI) dbEST and LifeSeq (Incyte Pharmaceuticals, Inc.) human EST databases for related sequences. When we examined expression of transcripts corresponding to the ESTs by

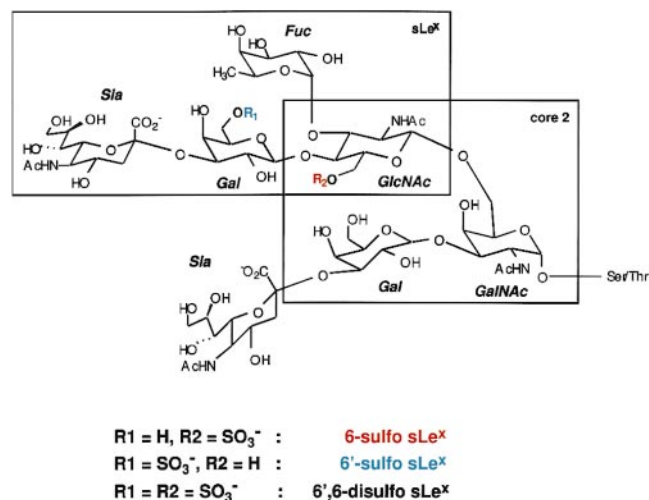


Figure 1. Sulfated O-linked carbohydrate chains of GlyCAM-1. Oligosaccharides bearing the 6-sulfo sLe^x and the sialyl 6'-sulfo Le^x motif, which extend from the core 2 structure, are shown. The presence of the sialyl 6',6-disulfo Le^x motif is strongly suspected. Structures of the more complex O-linked chains of GlyCAM-1 remain to be determined.

Northern analysis, one (corresponding to LifeSeq clone no. 2620445, derived from a human breast epithelial cell line) was of particular interest, because its tissue distribution was highly restricted (original finding not shown, but result demonstrated in Fig. 2 B). To determine whether this gene was expressed in HECs, a cell type in which L-selectin ligands are elaborated, we carried out a semiquantitative RT-PCR analysis on HEC cDNA with primers derived from this EST. HECs were purified, as described previously, from human tonsils using immunomagnetic separation with MECA-79 as the probe (Girard and Springer, 1995b; Sassetti et al., 1998). The resulting cells had a purity of >99% and represented <1 per 1,000 of the stromal cells in tonsils. In parallel, we prepared cDNA from HUVECs and tonsillar lymphocytes. As shown in Fig. 2 A, the primers specific for the novel gene amplified a fragment of 456 bp from HEC cDNA, but failed to amplify this product from the HUVEC or lymphocyte cDNA.

Since we had a relatively small number of HECs available, we used a PCR-based technique (SMART technology; Clontech, Inc.) to produce cDNA from total RNA, from which we prepared a plasmid expression library. Using PCR amplification of the 456-bp fragment to identify positive pools, we isolated a full-length cDNA clone from the library by a pool selection procedure, as described in Materials and Methods. Initially, 200 pools of 2,000 colony-forming units/pool were screened. After two additional rounds of screening, a single positive clone was obtained. The cDNA corresponding to this clone contains a single open reading frame of 1,158 nucleotides. The cDNA is apparently full-length as indicated by the presence of an upstream stop codon and a Kozak sequence surrounding the first ATG, and a poly(A) tail at the 3' end. This open reading frame predicts a type II transmembrane protein of 386 amino acids with 3 potential sites for N-linked glycosy-

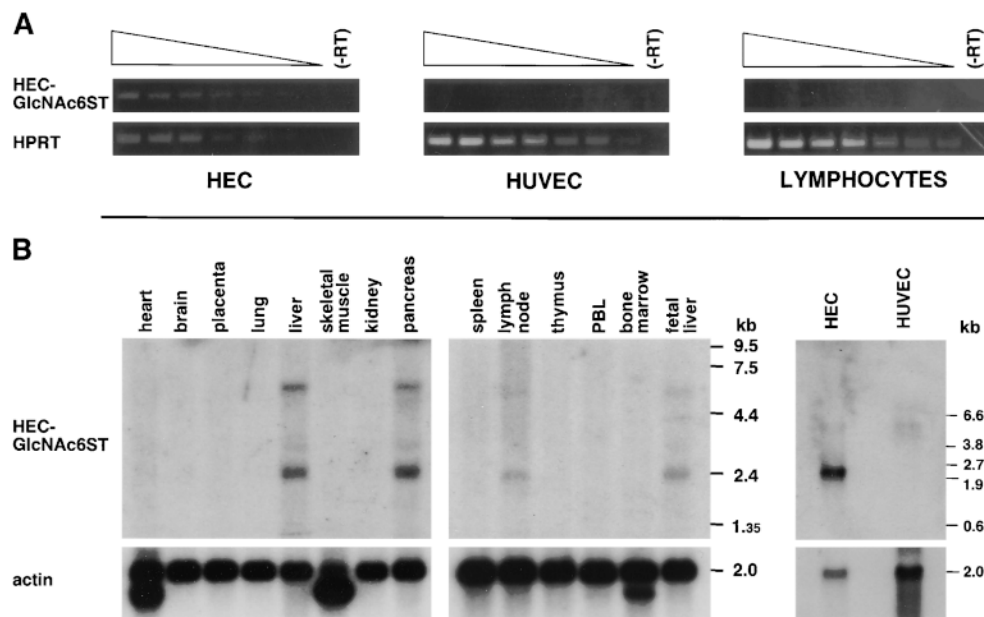


Figure 2. Expression of HEC-GlcNAc6ST transcripts in HECs. (A) Semiquantitative RT-PCR analysis. Fragments of the HEC-GlcNAc6ST and HPRT sequences were amplified by PCR from serial dilutions of cDNA prepared from purified HECs, HUVECs, and tonsillar lymphocytes. The reaction products (456 and 300 bp, respectively) were analyzed by agarose electrophoresis and ethidium bromide staining. -RT, PCR reactions in which the template was generated by omission of RT. (B) Northern blotting. Northern blots containing poly(A)⁺ RNA from various human tissues (left and center) and from

HECs and HUVECs (right) were probed with a 500-bp fragment from the HEC-GlcNAc6ST cDNA (top panels). The blots were stripped and reprobed with a 300-bp probe for β -actin (bottom panels).

lation. The new cDNA sequence was used to probe the human databases for additional matching ESTs. One EST was identified in the LifeSeq database that mapped to the new gene at the 5' end of its protein coding region. When the clone (no. 2617407, derived from gall bladder) corresponding to this EST was fully sequenced, its sequence completely matched the original cDNA sequence within the coding region. There were two base changes in the 3' untranslated region and divergence in the 5' untranslated region. We present the sequence corresponding to LifeSeq clone no. 2617407 (Fig. 3 A; EMBL/Genbank/DBJ accession no. AF131235), since the library from which it was cloned was created without a PCR amplification step. The predicted amino acid sequence of this novel gene is 31% identical to C6/KSST (Fukuta et al., 1995), the chicken enzyme used in the original EST search. We have termed this novel gene HEC-specific GlcNAc-6-sulfotransferase (HEC-GlcNAc6ST) on the basis of the characterization described below.

Using the human HEC-GlcNAc6ST cDNA sequence as a probe, we identified a highly related mouse sequence in the NCBI dbEST database (EMBL/GenBank/DBJ accession no. AA522184). A 241-bp probe based on this EST was used for screening of a BAC library from mouse (C57BL/6). The probe was found to hybridize to a single BAC. The genomic clone within this BAC contained an open reading frame of 1167 bp (EMBL/Genbank/DBJ accession no. AF131236), which is 77% identical at the nt level to the coding region of the human HEC-GlcNAc6ST. No introns were detected. The sequence predicts a protein of 388 amino acids (Fig. 3 A), which is 73% identical to that of human HEC-GlcNAc6ST. We term this apparent mouse homologue mHEC-GlcNAc6ST.

The expression of HEC-GlcNAc6ST was confirmed to be highly restricted by further analysis. On a conventional

Northern blot (human), transcripts corresponding to this gene were absent from most tissues (Fig. 2 B). Low levels of a 2.4-kb transcript were apparent in lymph node, liver (adult and fetal), and pancreas. A prominent band of the same size was detected at relatively high levels in mRNA from HECs, but was undetectable in HUVEC mRNA (Fig. 2 B). An additional transcript at ~6 kb was expressed in liver and pancreas, and trace levels appeared to be present in lymph node, HECs, and HUVECs (Fig. 2 B). Using an antisense riboprobe based on the original mouse EST, we carried out in situ hybridization on mouse tissues. Strikingly, mHEC-GlcNAc6ST transcripts were detected only in the HEVs of lymph node (Fig. 4). No hybridizing signal was found in other cell types of the lymph node, or in several other organs, including spleen, thymus, liver, skeletal muscle, pancreas, stomach, and kidney (data not shown). A weak signal was detected in gut intestinal epithelium (data not shown). The sense control did not yield signal in any tissue.

Our screening of the human EST databases with the chicken C6/KSST led to the identification of multiple ESTs that mapped to another gene. We cloned a cDNA for this gene by conventional techniques from a fetal brain library as described in Materials and Methods. While this work was in progress, the same cDNA (with only two base changes in untranslated regions) was published independently by Fukuta et al. (1997). The expressed protein was characterized as a keratan sulfate sulfotransferase with Gal-6-sulfotransferase activity (KSGal6ST) (Fukuta et al., 1997). The predicted protein is a type II transmembrane protein of 411 amino acids with 32% identity to HEC-GlcNAc6ST. Fukuta et al. (1997) reported the expression of KSGal6ST in many tissues, including lymph node. We confirmed these results; in addition, we detected its expression in HECs, albeit at apparently low levels, by per-

ccacgagcgcacgaaggctcttctgtggggaaatcttccttaatttgctttctccagcgcaactcaagcagctctccc	80
cacctctgagttcacagctcttaaagtctttaactccaagctctctggagcgagctgctcttcaaagcctgtctct	160
aaggtcttccactcaagcacaatCTACGTCGGTAAAAAATGAAGCTCCTCGTGTTTCTGGTTCCGAGATGCCACTT	240
M L L P K K M K K I T L F L V S G M A C T	20
M M L L L K K G R I T L N F P L Q T W V Y H	20
GGCTCTATTCTTCCACATGTACGACCAACAATCAGCTCGCTCTGTATGACGACGACGCCGAGCGATGCAGCTGTG	320
A L F E H M Y S V H N T S S L S M K A Q P E R M H V L V	47
A L F E H M S V S H R K H L S Q R E S R - R F V H V L V	46
TTCGTCTTCTCGCGCTCTGCGTCTCTTCTTGTGGGCGAGCTTTTGGGACGACCAGATGTTTCTACCTGATGG	400
L S S W R G S S G S S F Q L F G Q H D F V F Y L T	73
L S S W R G S S F V G Q L F G Q H D F V F Y L T	72
CCCCGCTGCGACCTGTGGTAGACCTTCAAGCAGACGACCCCTGGATCGCATGGCTGTGGGAGTCTGATACGGCG	480
P A W H V W M T F T K S T A W M L H M A V R D L I R A	100
P A W H V W M T F T K S T A W M L H M A V R D L I R A	99
CGTCTCTTGTGGCAGATCAGCGCTCTTTGATGCTCAATGGAACCTGGTCGCCGGAGACGCTCAGCGCTCTTACGTTGG	560
V F L C D M S V F D A Y M E P G A R R G S S I F Q W E	127
V F L C D M S V F D A Y M E P G A R R G S S I F Q W E	126
AGAACAAGCGGGCCCTGCTCTTGACACTGTGTGACATCTCCCAAGAAGTAATCATCCCGCGGCTCACTGCAGC	640
N S R A L C S A P A C D I P D E I I P R A H C R	153
N S R A L C S A P A C D I P A M K I I S P R H C K	152
CTCTCTCGACGTCACAAACGCTTGTAGGTTGGTGGAGAGSGCTCGCGCTCTTACAGCACAGCTGGTGTCAAGGAAGTGG	720
L C S S Q C O P P E T F V E K A C R S H V L V K E V	180
L L C G C O P P E T F V E K A C R S H V L V K E V	179
CTTCTTCAAGCTGCAGTCCCTCTACCGCGTGTGAAGAACCOCCTCCATGCATTCGATCTCGACACTGCTCGCGGACC	800
F F L N L Q A L Y P L L T D P S L N L H V V H L V R D P	207
F F L N L Q A L Y P L L T D P S L N L H V V H L V R D P	206
CCCCGGCGGTGTCGTCTCCGAGAACGACGCAAGGAGAGTCTCATGATTGACAGTCGATTGTGTGATGGGCGACATG	880
R A V F R S R R E T K G D L M V D S R I V G A Q H E	233
R A V F R S R R E T T T L T L M V D S H I V L G Q H E	232
CAGAAAACCTAAGAAAGAGGACGACCTACTATTGTATGACGAGTCACTGCCAAAGACGCTGAGGATCTACAAGACCAT	960
Q K L K K E D O P Y Y M Q V I C Q S Q L E I V K T I	260
E T I K E E D O P Y Y A M K I I C K S Q V L E I V K A I	259
CGACGTCTGCCCAAGGCGCTCGAGGAAGCATTCTGCTTGTGCGTCTATGAGGACCTGGCTCAGACPCTGTGGCCAGA	1040
Q S L P L K A L Q E R Y L L F L R Y E D L R A R A P A L Q T	287
Q S L P L K A L Q E R Y L L F L R Y E D L R A R A P A L Q T	286
CTTCCGATGATGAATACTGTGGGATTGGAAATTTCTGCCCACTTTCAGACCTGGGTGCATATACATACCCUAGGAGUC	1120
S R M Y E F V G L D F L P H L Q T I W H V N I T R G K	313
S R M Y E F V G L D F L P H L Q T I W H V N I T R G K	312
GGCATGGGTGACACGCTTTCACACAANAATCCAGGAGTGCCCTTAATGTCCTCCAGGCTTGGCGCTGGTCTTGGCCATA	1200
G M G G D R A F H T N A R D A L R N A L L V S Q A W R S L P Y	340
G M G G D R A F H T N A R D A L R N A L L V S Q A W R S L P Y	339
TGAAAATTTTTCGACTTCAGCAACCTTGGGCGATGCCAIGAAITTTGTGGCTACCCUACWRCAGCATCTGAACAGC	1280
E K V S R L Q R A C G D A M N L G Y L R H V R S Q E	367
E K V S R L Q R A C G E A M D L L G Y L R V R S Q E	366
AACGAGAAACATGTTGCTGGATCTCTGTCTACTCGGATCTCCCTGAGCAATFACCATAgagggttgagaaggtctt	1360
Q R N L L D L L S T T V T F E Q I H *	386
Q C C N L S L D L S S H I L G Q V A R E T G *	388
gclcgccgttgttcagctctcagctcattcttctaagctcttgagctctgcatcattctgagctcttaactacatgt	1440
tctctgggttagcactgagttgagttggtgccaccagctgtcaagcagacagttgtgtgtctcattgctgtgtctgag	1520
aaaacagactcaaacagctgagttgaggtgagcagctccccagctggaacaagaggttatgctctctcattgctgtgtgt	1600
ctcttgagagacagctgagctgagctgagctgagctgagctgagctgagctgagctgagctgagctgagctgagct	1680
acctctccttgccacctcttgcacatggggatggatcttccaacaaagctcagcagcatttccaacagatgcacaa	1760
tctgagcctctggagtccaagcagatccaagagagagggagacacatttgagctactatgatgattgaacat	1840
cagacgtactcgtatacagaatatgawacacattctctgcacacaaagagcagcagctcttaagtcaagcagcaggttcgct	1920
tgattgattgattcctcagctcagctcagctctctccactacataagagagcttgagctgagcagctgagctctgattac	2000
tatatccattcccaatgcgaacaaaaaanaaaaaa	2032

B

HEC-GlcNAc6ST
GlcNAc6ST
K3GAL6ST
C6ST

106 134

HEC-GlcNAc6ST
GlcNAc6ST
K3GAL6ST
C6ST

249 270

HEC-GlcNAc6ST
GlcNAc6ST
K3GAL6ST
C6ST

320 339

forming RT-PCR with HEC cDNA and the HEC-cDNA library (data not shown).

Sulfation of GlyCAM-1 by KSGal6ST and HEC-GlcNAc6ST

To test whether KSGal6ST and HEC-GlcNAc6ST (human) were capable of sulfating an L-selectin ligand, we transfected COS cells with a cDNA encoding a GlyCAM-

1/IgG chimera and a cDNA encoding one or the other sulfotransferase. The transfected cells were cultured in the presence of [35 S]sulfate, and radiolabeled GlyCAM-1/IgG was purified from the CM on protein A-agarose. As shown in Fig. 5 A, there was substantial incorporation of radioactivity into GlyCAM-1/IgG when either KSGal6ST or HEC-GlcNAc6ST cDNA (but not empty vector) was included in the cotransfection.

To establish the regiochemistry of sulfation on radiolabeled GlyCAM-1/IgG, samples resulting from the two sulfotransferase transfections were subjected to hydrolysis and Dionex HPLC analysis according to our previously established procedures (Hemmerich et al., 1994a). As shown in Fig. 5 B, transfection with KSGal6ST resulted in sulfated mono- and disaccharides that comigrated with $[\text{SO}_3 \rightarrow 6]\text{Gal}$ and $[\text{SO}_3 \rightarrow 6]\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}$, confirming this enzyme as a Gal-6-sulfotransferase (Fukuta et al. 1997). In contrast, transfection with HEC-GlcNAc6ST resulted in products that corresponded to $[\text{SO}_3 \rightarrow 6]\text{GlcNAc}$ and $\text{Gal}\beta 1 \rightarrow 4[\text{SO}_3 \rightarrow 6]\text{GlcNAc}$. Thus, this enzyme was established to be a novel GlcNAc-6-sulfotransferase.

Generation of Sialyl 6-sulfo Le^x with HEC-GlcNAc6ST

To determine whether the HEC-GlcNAc6ST could contribute to the generation of sialyl 6-sulfo Le^x (Table I), we took advantage of the G72 mAb, which recognizes this structure in a sulfate-dependent manner (Mitsuoka et al. 1998). We carried out transfection experiments with CHO cells, termed CHO/FTVII/C2GnT, which had been stably transfected with (a) fucosyltransferase VII (FTVII) to provide $\alpha 1 \rightarrow 3$ Fuc, and (b) core 2 $\beta 1 \rightarrow 6$ N-acetylglucosaminyltransferase (C2GnT) (Bierhuizen and Fukuda, 1992) to provide a core structure for O-linked glycans (Fig. 1) upon which extended chains with sLe^x capping groups can be elaborated (Li et al., 1996). The modified CHO cells were transiently transfected with HEC-GlcNAc6ST or KSGal6ST cDNA as a control. Transfection of the CHO cells with HEC-GlcNAc6ST cDNA resulted in significant staining by G72 as measured by flow cytometry (Fig. 6), whereas transfection with KSGal6ST did not yield staining (not shown). Similar results were obtained with G152 (Mitsuoka et al., 1998), a related mAb (data not shown). Inclusion of a CD34 cDNA in the transfection did not significantly enhance staining by G72, indicating that an exogenous mucin scaffold was not required for the expression of this epitope.

Contribution of KSGal6ST and HEC-GlcNAc6ST to the Generation of L-selectin Ligand Activity

Since HEC-GlcNAc6ST and KSGal6ST are both expressed

Table I. Nomenclature and Structure of Oligosaccharides

Name	Structure
sLe ^x	Siaα2→3Galβ1→4[Fuca1→3]GlcNAc
Sialyl 6'-sulfo Le ^x	Siaα2→3[SO ₃ →6]Galβ1→4[Fuca1→3]GlcNAc
Sialyl 6-sulfo Le ^x	Siaα2→3Galβ1→4[Fuca1→3][SO ₃ →6]GlcNAc
Sialyl 6',6-disulfo Le ^x	Siaα2→3[SO ₃ →6]Galβ1→4[Fuca1→3] [SO ₃ →6]GlcNAc
Core 2	Galβ1→3[GlcNAcβ1→6]GalNAc
6',6-disulfo lactose	[SO ₃ →6]Galβ1→4[SO ₃ →6]Glc

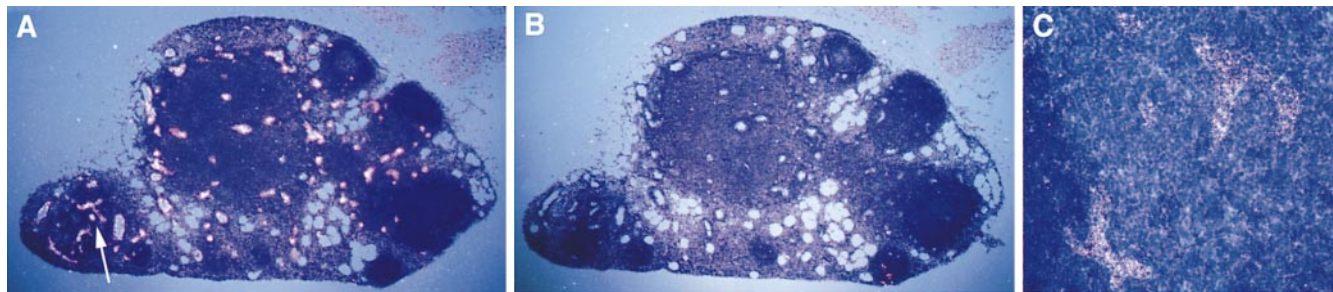


Figure 4. In situ hybridization to detect HEC-GlcNAc6ST transcripts in mouse lymph node. Sections of C56BL/6 mouse lymph node were hybridized with ^{35}S -labeled sense or antisense riboprobes based on the clone corresponding to the mouse homologue of HEC-GlcNAc6ST. Dark field micrographs of the sections are shown. Signal is seen as bright dots. (A) Hybridization with antisense probe, whole lymph node shown. The only source of signal are HEVs, seen as distinctive high-walled vessels in the cortex of the node. (B) Hybridization with sense probe of section adjacent to that in A. (C) Higher magnification view of area indicated by arrow in A. Two large HEVs are evident.

in HECs and are capable of sulfating GlyCAM-1/IgG in transfected cells, they are candidates to participate in the biosynthesis of L-selectin ligands. Our previous structural analysis indicated that Gal-6-sulfate and GlcNAc-6-sulfate are present equally in native GlyCAM-1 oligosaccharides. However, as reviewed above, the relative contribution of the two sulfate esters to L-selectin binding affinity has been a matter of considerable uncertainty. To test whether these enzymes can contribute to the generation of ligand activity, we carried out further flow cytometry experiments with an L-selectin/IgM chimera as a probe. The CHO/FTVII/C2GnT cells were transiently transfected with cDNAs for the two sulfotransferases (singly or in combination) plus a CD34 cDNA. As shown in Fig. 7 A, no binding of the chimera was detected in the absence of the sulfotransferases. Transfection with either sulfotransferase yielded positive staining, both in terms of the proportion

of positive cells and their mean fluorescence intensity (MFI). Strikingly, the combination of KSGal6ST and HEC-GlcNAc6ST cDNAs produced strongly enhanced binding of L-selectin/IgM, which greatly exceeded the sum of the signals from the individual sulfotransferases (Fig. 7 A). This apparent synergistic effect was evident over a considerable range of cDNA concentrations in the transfection mixtures (Tables II and III). The binding of the L-selectin/IgM chimera induced by the sulfotransferases was confirmed to be specific as indicated by its calcium dependence (data not shown) and complete inhibition binding by a function-blocking anti-L-selectin mAb (MEL-14; Fig. 7 B). The effects of the sulfotransferases on the L-selectin/IgM chimera were not due to differences in transfection efficiency, as the CD34 expression levels were essentially constant, irrespective of the combinations of sulfotransferase cDNAs in the transfection mixtures (Table II). Fur-

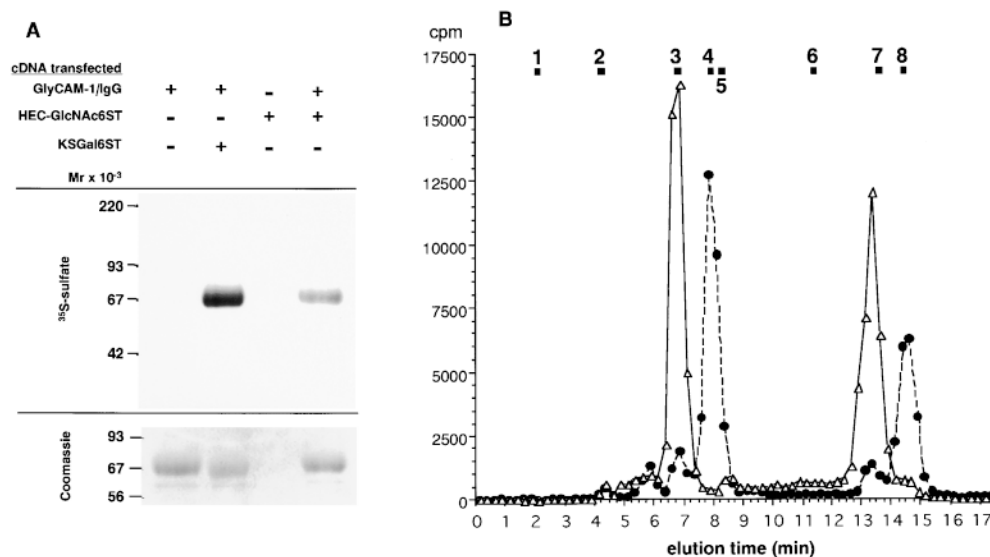


Figure 5. Sulfation of GlyCAM-1/IgG by sulfotransferases. COS cells were transfected with combinations of plasmids encoding GlyCAM-1/IgG, KSGal6ST, and HEC-GlcNAc6ST, as indicated. Transfected cells were cultured in the presence of ^{35}S sulfate, and recombinant GlyCAM-1/IgG was isolated from the CM. 1% of the captured material was analyzed by SDS-PAGE, and the remainder was subjected to hydrolysis and compositional analysis. (A) Autoradiograph (top) and Coomassie Blue staining (bottom) of SDS gel. Densitometric quantification of the Coomassie Blue-stained

bands showed that each lane, except the control lane without GlyCAM-1/IgG plasmid, contained approximately equal amounts of GlyCAM-1/IgG. (B) Sulfated carbohydrates produced in GlyCAM-1 by transfection with HEC-GlcNAc6ST (Δ) or KSGal6ST (\bullet) were analyzed by Dionex chromatography after acid hydrolysis. The following standards are indicated: 1, GlcNAc-3 SO_3^- ; 2, ^{35}S SO_4^{2-} ; 3, Gal β 1 \rightarrow 4[SO_3^- \rightarrow 6]GlcNAc; 4, [SO_3^- \rightarrow 6]Gal β 1 \rightarrow 4GlcNAc; 5, Gal-4 SO_3^- ; 6, Gal-3 SO_3^- ; 7, GlcNAc-6 SO_3^- ; 8, Gal-6 SO_3^- .

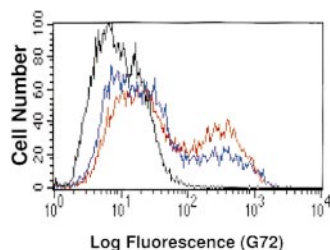


Figure 6. Generation of sialyl 6-sulfo Le^x by transfection with HEC-GlcNAc6ST cDNA. CHO/FTVII/C2GnT cells were transfected with a cDNA encoding HEC-GlcNAc6ST, with or without CD34 cDNA. Cells were stained with the G72 mAb to detect the presence of sialyl 6-sulfo Le^x. Histogram shows G72 staining for the transfections with (red) or without (blue) CD34 cDNA, or staining of the isotype control antibody for the transfection with CD34 cDNA (black).

thermore, to control for the possibility that the sulfotransferases might cause changes in essential glycosylation parameters that could affect L-selectin binding, we stained the transfected cells with the HECA 452 mAb. This antibody recognizes sLe^x-related structures and is widely used as a reporter for glycosylation modifications (sialylation and fucosylation) pertinent to selectin ligands (Wagers et al., 1997; Tu et al., 1999). Because this antibody reacts equally well with sulfated (at Gal-6, GlcNAc-6, or both) and nonsulfated sLe^x structures (Mitsuoka et al., 1998), it was of particular utility for detecting the overall presence of sLe^x on the cell surface of the transfectants. As shown in Table III, the expression of the HECA 452 epitope was not significantly altered (<25% variation) by transfection with the sulfotransferase cDNAs in any combination. The final issue we investigated was the contribution of CD34 to the expression of L-selectin ligand activity. In striking contrast to the results with G72, the binding of L-selectin/IgM was dependent on the presence of the CD34 protein scaffold, as there was a nearly complete loss of staining when the CD34 cDNA was omitted from the transfection (Fig. 7 C). Interestingly, among the CD34-positive population, only those cells expressing the highest levels stained with the L-selectin/IgM chimera (Fig. 7 A).

Table II. L-selectin/IgM and CD34 Staining of CHO Cells Transfected with Combinations of Sulfotransferase cDNAs

cDNA transfected		MFI	
KSGal6ST	HEC-GlcNAc6ST	L-sel/IgM staining	CD34 staining
μg			
0	0	0	660
1.0	0	364	742
1.5	0	391	731
0	1.0	114	748
0	1.5	163	700
0.5	0.5	935	743
1.0	0.5	917	684
0.5	1.0	830	703

CHO/FTVII/C2GnT cells were cotransfected with plasmids encoding CD34 (2 μg) and each sulfotransferase singly or in combination in the indicated amounts. Data are expressed as the mean fluorescence intensity (MFI) of L-selectin/IgM staining in the L-sel-IgM⁺/CD34⁺ population with background signal (from transfectants with CD34 cDNA alone, value 139) subtracted. Also shown is the MFI for staining with a CD34 mAb (Qbend-10) for the population that was positive for CD34, as defined by staining with the class-matched control antibody.

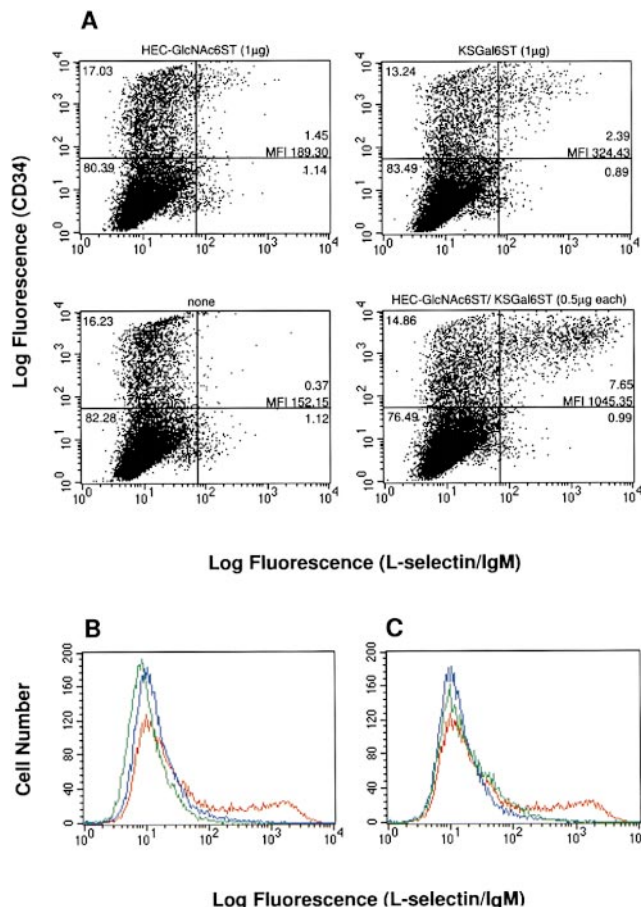


Figure 7. L-selectin reactivity conferred to CD34 by transfection with HEC-GlcNAc6ST and KSGal6ST cDNAs. CHO/FTVII/C2GnT cells were transfected with different combinations of cDNAs encoding human CD34, HEC-GlcNAc6ST, and KSGal6ST. Cells were stained with the L-selectin/IgM chimera to detect ligand activity. (A) Two-color analysis showing CD34 expression (y-axis, staining with CD34-PE mAb) and L-selectin ligand activity (x-axis, FITC) in cells transfected with CD34 cDNA and cDNAs encoding HEC-GlcNAc6ST and KSGal6ST, alone or in combination as indicated. The horizontal bar is set such that all cells staining with the isotype-matched control for the CD34 mAb are included in the lower quadrants. The vertical bar is set to indicate the L-selectin/IgM staining of cells in which no sulfotransferase cDNA was included in the transfection mixture (bottom left panel). The fraction of positive cells (as a percentage of the total) in each quadrant is indicated. The mean fluorescence intensity (MFI) for the cells in the top right quadrants is indicated. The CD34 mAb did not interfere with staining by the L-selectin/IgM chimera. (B and C) Histograms showing L-selectin/IgM staining for the following transfections: CD34 cDNA only (blue, B and C); CD34/HEC-GlcNAc6ST/KSGal6ST cDNAs (red, B and C); CD34/HEC-GlcNAc6ST/KSGal6ST cDNAs with staining done in the presence of Mel-14 mAb (green, B); HEC-GlcNAc6/KSGal6ST cDNAs but no CD34 cDNA (green, C). The secondary staining reagent when used alone showed staining equal to that observed when Mel-14 mAb was used as an inhibitor. The isotype-matched control for Mel-14 showed no inhibitory effect.

Discussion

Sulfation plays a central role in the interactions of L-selectin and P-selectin with their physiological ligands (Rosen

Table III. L-selectin/IgM and HECA 452 Staining of CHO Cells Transfected with Combinations of Sulfotransferase cDNAs

cDNA transfected		MFI	
KSGal6ST	HEC-GlcNAc6ST	L-sel/IgM staining	HECA staining
μg			
0	0	0	665
1.0	0	197	697
0	1.0	60	496
0.5	0.5	836	561

CHO/FTVII/C2GnT cells were cotransfected with plasmids encoding CD34 (2 μg) and each sulfotransferase singly or in combination in the indicated amounts. Data are expressed as the MFI of L-selectin/IgM staining in the L-sel-IgM⁺/CD34⁺ population with background signal (from transfectants with CD34 cDNA alone, value 182) subtracted. Also shown is the MFI for staining with the HECA 452 mAb for the entire population. The MFI for staining with a control antibody was <10.

and Bertozzi, 1996). PSGL-1, a leukocyte ligand for both P-selectin and L-selectin (McEver and Cummings, 1997), possesses a cluster of sulfated tyrosine residues, which facilitate binding of both selectins (Pouyani and Seed, 1995; Sako et al., 1995; Wilkins et al., 1995; Snapp et al., 1998). As reviewed above, L-selectin recognition of its cognate HEV ligands requires sulfation, as well as fucosylation and sialylation, for optimal binding. For the molecularly defined HEV ligands, sulfation is on sugar moieties rather than tyrosine residues (Rosen and Bertozzi, 1996). Our objective has been the molecular identification of the sulfotransferases that participate in the biosynthesis of these endothelial ligands. The specificities of the desired sulfotransferases were dictated by the analysis of the oligosaccharides of L-selectin-reactive GlyCAM-1, which revealed the presence of Gal-6-sulfate and GlcNAc-6-sulfate in the context of sLe^x (sialyl 6'-sulfo Le^x and sialyl 6-sulfo Le^x, respectively). Similarly, the functional glycoforms of CD34 also contain Gal-6-sulfate and GlcNAc-6-sulfate in approximately equal representations (Hemmerich and Rosen, unpublished observations). Histochemical evidence with carbohydrate-directed mAbs shows that sialyl 6-sulfo Le^x is prominently displayed on the HEVs of human peripheral lymph node (Mitsuoka et al., 1998), further implicating a GlcNAc-6-sulfotransferase activity in the biosynthesis of L-selectin ligands. Bowman et al. (1998) recently characterized such an activity from porcine lymph node, which was highly enriched in HECs.

To identify the relevant sulfotransferases at the molecular level, we probed human EST databases for homologues of the chicken C6/KSST, a bona fide Gal-6-sulfotransferase. Here we report the cloning of two carbohydrate sulfotransferases. One is indeed a Gal-6-sulfotransferase, which was independently discovered by Fukuta et al. (1997) and named KSGal6ST. The second enzyme, which we have termed HEC-GlcNAc6ST, is a novel GlcNAc-6-sulfotransferase. These two sulfotransferases, together with the chicken C6/KSST (Fukuta et al., 1995), and the recently reported human chondroitin-6-sulfotransferase (C6ST, specificity for C-6 of GalNAc) (Fukuta et al., 1998) and GlcNAc-6-sulfotransferase (GlcNAc6ST) (Uchimura et al., 1998a,b) constitute a family of highly conserved enzymes. Overall, amino acid identities within the family range from 27 to 42%. These enzymes are type II trans-

membrane proteins with short cytoplasmic tails, features that are typical of glycosyltransferases and carbohydrate sulfotransferases.

Within this new family of carbohydrate sulfotransferases, there are three regions of amino acid sequence in which amino acid identity ranges from 45 to 54% and similarity from 80 to 90% (Fig. 3 C). Regions one and two contain elements that conform to the recently described consensus binding motifs for the high energy sulfate donor, 3'-phosphoadenosine 5'-phosphosulfate. These elements are found in all sulfotransferases characterized to date (Kakuta et al., 1998). In addition, regions one and three contain two stretches of sequence of 11 amino acids each (corresponding to amino acids 124–134 and 328–339, respectively, in the HEC-GlcNAc6ST sequence) that are highly conserved (>90% similarity). It is possible that these two elements contribute to a binding pocket that interacts with the 6-hydroxyl group of an appropriate oligosaccharide acceptor (Gal, GalNAc, or GlcNAc) to bring it into apposition with the donor phosphosulfate group.

Our characterization of the KSGal6ST and HEC-GlcNAc6ST supports their involvement in the synthesis of L-selectin ligands. KSGal6ST has a wide tissue distribution which includes HECs. HEC-GlcNAc6ST shows a highly restricted, although not absolute, localization to HECs. Second, the sulfotransferases catalyze the two specific sulfation modifications on a recombinant L-selectin ligand that have been established to occur on native ligands. In the case of HEC-GlcNAc6ST, we have used the G72 mAb to show that transfection with the cDNA leads to 6-sulfation of sLe^x on the cell surface of CHO/FTVII/C2GnT cells. This finding establishes that recombinant HEC-GlcNAc6ST can be used to generate a highly specific sulfated structure that is present on lymph node HEVs (Mitsuoka et al., 1998).

Using a flow cytometry assay based on the binding of an L-selectin/IgM chimera to transfected CHO/FTVII/C2GnT cells, we showed that each enzyme imparts L-selectin binding. Control experiments established that the effects of the sulfotransferases were not due to indirect effects on transfection efficiencies or to global changes in glycosylation parameters of the cells. In contrast to the generation of the G72 epitope, L-selectin/IgM binding required cotransfection of the cells with a CD34 cDNA. These results indicate that a specific protein scaffold, not present endogenously in CHO cells, is needed for optimal ligand activity, although at least one of the relevant sulfated carbohydrate epitopes (sialyl 6-sulfo Le^x) can be formed without the provision of such a scaffold. This finding is analogous to the situation with P-selectin, in which sLe^x determinants can be formed by transfection of COS cells with an appropriate fucosyltransferase cDNA, but ligand activity requires the inclusion of PSGL-1 cDNA (Sako et al., 1993). As noted above, a shared feature of the molecularly defined HEV-associated ligands for L-selectin is the presence of a mucin region (Puri et al., 1995). This feature provides the potential for multivalent presentation of carbohydrate recognition determinants, which is thought to be important for enhancing the avidity of L-selectin interactions (Nicholson et al., 1998). Thus, the CD34 contribution to ligand activity seen in the present study may be primarily due to its mucin character. The ability of other

mucins to perform this postulated scaffold function and their possible distinct usage of the different sulfotransferases are questions for future investigation.

While each sulfotransferase was capable of conferring L-selectin binding onto CD34 in the CHO/FTVII/C2GnT cells, the greatest effect was clearly produced by the combined action of the two sulfotransferases. The level of binding achieved in the cells transfected with both sulfotransferases could not be attained with either sulfotransferase alone, over a wide range of cDNA concentrations. These findings argue that optimal binding to L-selectin requires both the Gal-6-sulfate and GlcNAc-6-sulfate moieties. It is possible that this synergy arises through dual recognition of separate monosulfated chains, for example one chain capped by sialyl 6-sulfo Le^x and the other by sialyl 6'-sulfo Le^x. This mechanism would fit a model of selectin binding proposed by Varki (1994), in which a specific cluster of adjacent O-linked chains comprises the full recognition determinant. Alternatively, individual chains containing both modifications may underlie the synergistic effect. In this regard, it should be noted that most of the sulfated chains within GlyCAM-1 contain two or more sulfates, although the structure of these chains has not been solved (Hemmerich et al., 1995). It is not yet clear whether sialyl 6',6-disulfo Le^x (Table I) exists as a capping group on these multisulfated chains or whether there are extended chains containing sulfates on internal Gal or GlcNAc residues. Additional analysis of natural ligands will be required to resolve these important structural questions.

L-selectin normally functions in tethering and rolling of lymphocytes on HEVs under conditions of blood flow (Warnock et al., 1998). Future experiments must determine the impact of the two sulfation modifications on the kinetic parameters of L-selectin binding to ligands, as these parameters are the key determinants of the dynamic interactions of lymphocytes with HEVs (Alon et al., 1997). We are currently addressing these issues using a parallel plate flow chamber to recapitulate physiological flow dynamics within blood vessels (Lawrence et al., 1995).

On the basis of its activities and highly restricted expression pattern, HEC-GlcNAc6ST is likely to be specifically involved in the biosynthesis of the sialyl 6-sulfo Le^x epitope and related structures within L-selectin ligands. Ultimately, it may be necessary to inactivate the gene in mice by gene knockout to define the biological roles of this enzyme. Experiments of this type were required to demonstrate the critical role for FTVII in the generation of selectin ligands, including the HEV ligands for L-selectin (Maly et al., 1996). We cannot rule out the possible involvement of another recently cloned GlcNAc-6-sulfotransferase identified in mice and humans (Uchimura et al., 1998a,b) in ligand biosynthesis. As revealed by *in situ* hybridization, transcripts encoding the latter enzyme were present in HECs of mouse lymph nodes, although the overall tissue expression pattern was extremely broad in both species, suggesting other functions as well. In this regard, it should be noted that 6-sulfated GlcNAc residues are present in the GAG chains of keratan sulfate and a variety of glycoproteins, including the N-linked oligosaccharides of thyroglobulin (Kamerling et al., 1988), gp120 of HIV (Shilatifard et al., 1993), and porcine zona pellucida

(Noguchi and Nakano, 1992). Furthermore, the GlcNAc-6-sulfate motif in the context of the core-2 structure has been identified in respiratory mucins from cystic fibrosis patients (Lo-Guidice et al., 1994), and a GlcNAc-6-sulfotransferase activity, which can act on mucins, has been detected in human respiratory mucosa (Degroote et al., 1997).

With respect to the sialyl 6'-sulfo Le^x epitope, our data on the acceptor specificity and expression pattern of KSGal6ST are consistent with a possible role for this enzyme in the generation of the epitope within HECs. However, this enzyme (Fukuta et al., 1997), like the GlcNAc-6-sulfotransferase described by Uchimura and colleagues (1998a,b), is broadly distributed, being expressed in all of the major organs. In analogy with HEC-GlcNAc6ST described in the present study, one might predict the existence of an HEC-specific, or more highly restricted, Gal-6-sulfotransferase that sulfates L-selectin ligands *in vivo*.

L-selectin-dependent leukocyte trafficking into lymphoid organs and inflammatory sites is likely to be subject to complex regulation, which may be affected at the level of L-selectin ligand biosynthesis through the availability of protein scaffolds or by posttranslational modification. Given the dramatic sulfate dependency of the interaction between L-selectin and its ligands, and the high [³⁵S]sulfate incorporation seen in HEVs and HEV-like vessels (Girard and Springer, 1995a), it is tempting to speculate that regulation of the expression or activity of one or both of the relevant sulfotransferases represents critical control points for this process. It is further plausible that the differential expression of the sulfotransferases may contribute to the apparent variation in the posttranslational modifications of L-selectin ligands in different lymphoid organs (Berg et al., 1998; Mitsuoka et al., 1998). The expression of KSGal6ST and HEC-GlcNAc6ST, as well as other candidate sulfotransferases, should also be examined in HEV-like vessels that are induced at sites of chronic inflammation (Michie et al., 1993; Girard and Springer, 1995a; Onrust et al., 1996). Endothelial ligands for L-selectin are also inducible on flat-walled vessels *in vivo*, although the biochemical nature of these ligands is poorly understood (Ley and Tedder, 1995). *In vitro* models of endothelial activation suggest that sulfation is required for the activity of these ligands (Giuffrè et al., 1997; Zakrzewicz et al., 1997; Tu et al., 1999). Endothelial sulfotransferases that are implicated in inflammatory leukocyte trafficking would clearly assume importance as potential targets for anti-inflammatory therapeutics.

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